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# The Broadly Neutralizing Anti-Human Immunodeficiency Virus Type 1 Antibody 2G12 Recognizes a Cluster of α1→2 Mannose Residues on the Outer Face of gp120

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**2G12 is a broadly neutralizing human monoclonal antibody against human immunodeficiency virus type-1 (HIV-1) that has previously been shown to bind to a carbohydrate-dependent epitope on gp120. Here, site-directed mutagenesis and carbohydrate analysis were used to define further the 2G12 epitope. Extensive alanine scanning mutagenesis showed that elimination of the N-linked carbohydrate attachment sequences associated with residues N295, N332, N339, N386, and N392 by N→A substitution produced significant decreases in 2G12 binding affinity to $gp120_{JR\text{-}CSF}$. Further mutagenesis suggested that the glycans at N339 and N386 were not critical for 2G12 binding to $gp120_{JR\text{-}CSF}$. Comparison of the sequences of isolates neutralized by 2G12 was also consistent with a lesser role for glycans attached at these positions. The mutagenesis studies provided no convincing evidence for the involvement of gp120 amino acid side chains in 2G12 binding. Antibody binding was inhibited when gp120 was treated with *Aspergillus saitoi* mannosidase, Jack Bean mannosidase, or endoglycosidase H, indicating that Manα1→2Man-linked sugars of oligomannose glycans on gp120 are required for 2G12 binding. Consistent with this finding, the binding of 2G12 to gp120 could be inhibited by monomeric mannose but not by galactose, glucose, or *N*-acetylglucosamine. The inability of 2G12 to bind to gp120 produced in the presence of the glucose analogue *N*-butyl-deoxynojirimycin similarly implicated Manα1→2Man-linked sugars in 2G12 binding. Competition experiments between 2G12 and the lectin cyanovirin for binding to gp120 showed that 2G12 only interacts with a subset of available Manα1→2Man-linked sugars. Consideration of all the data, together with inspection of a molecular model of gp120, suggests that the most likely epitope for 2G12 is formed from mannose residues contributed by the glycans attached to N295 and N332, with the other glycans playing an indirect role in maintaining epitope conformation.**

The humoral immune response to infection by human immunodeficiency virus type 1 (HIV-1) is typically characterized by relatively low levels of neutralizing antibodies, particularly those with broad activity against many different isolates of the virus (6, 23, 35, 36, 46). As might perhaps be anticipated from this observation, the induction of such antibodies by vaccination has proven largely elusive (9). At the same time, interest in inducing broadly neutralizing antibodies has increased as it becomes clear that antibodies can provide considerable benefit against HIV or simian immunodeficiency virus (SIV) challenge in animal models (1, 17, 30–32, 44, 55). Fortunately, natural infection is not completely barren of lessons for vaccine design since, although HIV elicits weak cross-neutralizing responses, a small number of human monoclonal antibodies (MAbs) with broad activities have been isolated from infected individuals (7, 8, 12, 59, 60). One rational contribution to eliciting neutralizing antibodies by vaccination is then to explore the interaction of these antibodies with virus envelope at the molecular level and incorporate the information obtained into immunogen design. Here, we seek to understand the interaction of one broadly neutralizing antibody with HIV-1 envelope.

For some time, only three broadly neutralizing MAbs to HIV-1 were known (5, 12). Two of these MAbs bind to the surface glycoprotein, gp120, which is the viral receptor for CD4 and chemokine receptors CCR5 and CXCR4. These MAbs are b12, which recognizes an epitope overlapping the CD4 receptor site (7, 51), and 2G12, which recognizes an epitope based around the C4/V4 region of gp120 and is highly sensitive to the presence of N-linked glycans in this region (24, 60). One MAb, 2F5, binds to an epitope involving a linear motif (ELDKWA) on the membrane proximal region of the transmembrane envelope protein gp41 (8, 24, 43, 71). Recently, two MAbs, Z13 and 4E10, have been described which recognize a region close to the C terminus of the 2F5 epitope (56, 71). Another Fab with broad neutralizing ability, X5, recognizes a region close to the coreceptor binding site on gp120 and overlapping the epitope recognized by CD4-induced MAbs, such as 17b (37a).

We focus here on the MAb 2G12. In vitro, this MAb has been shown to neutralize a wide spectrum of different HIV-1 isolates (59, 60), including those from different clades, with the

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notable exception of clade E. In vivo, the MAb protects macaques against vaginal challenge with the chimeric virus SHIV 89.6P (32). The antibody recognizes a unique epitope in that it does not compete with any of the large panel of MAbs to gp120 that have been produced (37). The binding of 2G12 to gp120 is inhibited by a number of mutations that disrupt sequences encoding attachment of N-linked carbohydrate chains (60). These sequences are located in the C2 and C3 regions around the base of the V3 loop, the C4 region, and the V4 loop. The crystal structure of the core of gp120 suggests that the carbohydrate attachment sites are clustered together on a part of the gp120 molecule known as the "silent face" (27, 28, 66, 67). This extensive solvent-accessible face is largely covered by carbohydrate and expected to be relatively weakly immunogenic and, hence, is described as immunologically silent.

Carbohydrate-rich regions of glycoproteins are generally poorly immunogenic for a number of reasons. First, carbohydrates exhibit microheterogeneity; therefore, a single protein sequence would be expected to display multiple glycoforms, leading to the dilution of any single antigenic response (52). Second, large, potentially dynamic glycans (62, 64) can cover potential protein epitopes. Third, in the case of viruses, which depend on the host glycosylation machinery since they have none of their own, the oligosaccharides attached to potential antigens are the same as those attached to host glycoproteins. Therefore, in general, the host will display tolerance towards these sugars. The difficulty in eliciting antibodies to a carbohydrate face is consistent with the apparently unique nature of MAb 2G12.

Despite the probable placement of the 2G12 epitope on the silent face (or at the junction of silent and neutralizing faces) (26, 27, 49, 60), we understand relatively little about the molecular nature of the epitope. We do not know whether the epitope is exclusively carbohydrate, exclusively protein with a requirement for carbohydrate to maintain local protein structure, or some combination of these. We do not know the relative importance of the different carbohydrate chains that are potentially involved or which sugar residues are likely to be crucial.

To address these issues we have carried out a number of studies on the 2G12-gp120 interaction. These include a detailed glycan analysis of the gp120 N-linked carbohydrates, an examination of the effects of digestion of gp120 by various glycosidases, an analysis of the ability of various glycans and lectins to inhibit the interaction, extensive alanine scanning mutagenesis of gp120, sequence comparisons of gp120s with different abilities to interact with 2G12 and, finally, modeling studies of gp120. We conclude that Man$\alpha$1$\rightarrow$2Man-linked residues of the outer face of gp120 are required for the 2G12 epitope. Extensive site-directed mutagenesis demonstrated little dependence of 2G12 affinity on specific gp120 amino acid side chains.

## MATERIALS AND METHODS

**Plasmid constructs and mutagenesis.** Alanine point mutations (Table 1) were generated using the QuickChange mutagenesis kit (Stratagene) with plasmid pSVIIIexE7pA$^-_{JR\text{-}CSF}$ (72) as the template. A V1 loop-deleted mutant (deletion of residues 134 to 154) was generated using plasmid pSVIIIexE7pA$^-_{JR\text{-}CSF}$ with the primer pair csf120-f (5′-GTCTGAGTCGGAGCTAGCGTAGAAAAGTTG TTGGGTCA-3′) and csfdV1-r (5′-GTCTGAGTCGGAACCGGACCCATCTT TGCAATTTAAAGTA-3′) and the primers csfdV1-f (5′-GTCTGAGTCGGAT CCGGTTCTGGGAAAAACTGCTCTT-3′) and csf120-r (5′-GTCTGAGTCG GACTCGAGTTTTCTCTTTGCACCACTCTTC-3′). Primers csfdV1-f and csfdV1-r both contain a unique *Bsa*WI restriction site. The PCR products were cloned into pSVIIIexE7pA$^-_{JR\text{-}CSF}$ using *Kpn*I, *Bsa*WI, and *Mfe*I in a two-step ligation reaction. The V3 loop-deleted mutant (deletion of 303 to 324) was generated similarly, using the primers csf120-f and csfdV3-r (5′-GTCTGAGTC GGSSCCGGACCCATTGTTGCTGGGCCTTGT-3′) and primers csfdV3-f (5′-GTCTGAGTCGGATCCGGTTCTGGGGATATAAGACAAGCCC-3′) and csf120-r. Primers csfdV3-f and csfdV3-r also contain a unique *Bsa*WI restriction site. To generate a V1/V2 loop-deleted mutant (V2 loop deleted from residues 160 to 193), the pSVIIIexE7pA$^-_{JR\text{-}CSF}$-$\Delta$V1 mutant was used as a template. First, the introduced *Bsa*WI site was mutated, whereby the amino acid sequence was retained. Deletion of the V2 loop sequences was performed in an analogous manner as for the other two mutants, using primers csf120-f and csfdV2-r (5′-GTCTGAGTCCGAACCGGACCCGAAAGAGCAGTTTTT-3′) and primers csdV2-f (5′-GTCTGAGTCGGATCCGGTTCTGGGATAAGTTGTAACA CC-3′) and csf120-r. PCR fragments were cloned into pSVIIIexE7pA$^-_{JR\text{-}CSF}$ using *Kpn*I, *Bsa*WI, and *Mfe* I. In all variable loop-deleted mutants, the deleted sequences were replaced by a GSGSG linker. All mutations generated in this study were verified by DNA sequencing.

**Generation of recombinant HIV-1 virions.** To produce recombinant virions, 293T cells grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with penicillin, streptomycin, L-glutamine, and fetal bovine serum (10%) were transiently transfected with mutant or wild-type plasmids (2 $\mu$g) along with plasmid pNL4.3LucR$^-$E$^-$ (4 $\mu$g; obtained from the National Institutes of Health AIDS Research and Reference Reagent Program), using FuGENE transfection reagent (Roche) according to the manufacturer's instructions. At 24 h posttransfection, the culture supernatant was replaced with serum-free medium and incubation was continued for another 24 h. The culture supernatants were harvested, and recombinant virions were lysed by the addition of detergent. The samples were stored at $-20$°C until further use.

**Enzyme-linked immunosorbent assays (ELISAs).** To determine the relative binding affinity of 2G12 for wild-type and mutant envelope glycoproteins, microtiter plate wells (flat bottom, Costar type 3690; Corning Inc.) were coated overnight at 4°C with anti-gp120 antibody D7324 (International Enzymes Inc.) at a concentration of 5 $\mu$g/ml (diluted in phosphate-buffered saline [PBS]). Subsequent incubation steps were performed at room temperature. Coated plates were washed twice with PBS supplemented with 0.05% Tween (PBS-T), blocked for 1 h with PBS supplemented with 3% bovine serum albumin (BSA), and subsequently incubated for 4 h with cell culture supernatants diluted 1:3 in PBS containing 1% BSA and 0.02% Tween (PBS-B-T). Plates were washed with PBS-T (10 times) and then incubated with MAb serially diluted in PBS-B-T (starting at a concentration of 10 $\mu$g/ml). Purified immunoglobulin G (IgG) from HIV-positive patients (1 $\mu$g/ml, diluted in PBS-B-T) was used as a control to ensure that similar amounts of envelope protein were captured. After washing as before, peroxidase-conjugated goat anti-human IgG [F(ab′)$_2$ specific; Pierce], was added (diluted 1:1,000 in PBS-B-T), and incubation continued for another hour. Plates were washed again, followed by incubation with TMB substrate (Pierce). The color reaction was stopped by adding 2 M sulfuric acid, and the optical density was measured at 450 nm. Apparent affinities were calculated as the antibody concentration at 50% maximal binding; changes in affinity were expressed as [(apparent affinity of wild type)/(apparent affinity of mutant)] $\times$ 100%.

gp120 with modified glycosylation was obtained by incubating recombinant gp120$_{JR\text{-}FL}$ (1 $\mu$g; gift from Bill Olson and Paul Maddon) at 37°C in the presence of either *Aspergillus saitoi* mannosidase (20 $\mu$U; 72 h), Jack Bean mannosidase (3 U; 24 h), or endoglycosidase H (endoH; 40 mU; 24 h) in 10 $\mu$l of the manufacturer's recommended buffer (Glyko Inc.). Antibody affinity was determined as described above; glycosidase- or mock-treated gp120$_{JR\text{-}FL}$ (0.1 $\mu$g/ml) was captured onto antibody-coated plates for 1 h at room temperature, prior to adding antibody. The binding affinities of MAb b12 and cyanovirin (CVN), an 11-kDa bacterial lectin which reacts with the $\alpha$1$\rightarrow$2 mannose residues of gp120 oligomannose structures (2–4, 13), for gp120 with modified glycosylation were assayed in an analogous manner, except that CVN binding was detected using a rabbit anti-CVN antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (heavy- and light-chain specific; diluted 1:1,000 in PBS-B-T; Pierce) and *p*-nitrophenyl phosphate (Sigma) as a substrate. For CVN, optical density was measured at 405 nm.

For 2G12 inhibition experiments, recombinant gp120 (0.1 $\mu$g/ml) was first captured onto microtiter plate wells with antibody D7234. 2G12 (serially diluted starting at a concentration of 10 $\mu$g/ml) was subsequently added in the presence of various concentrations of D-mannose (5, 50, and 500 mM) or in the presence

TABLE 1. Alanine and variable loop-deleted mutants used in this study and their effect on 2G12 binding

| Position in gp120[a] | Mutation[b] | Antibody affinity relative to wild type (%)[c] | Effect[d] | Position in gp120[a] | Mutation[b] | Antibody affinity relative to wild type (%)[c] | Effect[d] |
|---|---|---|---|---|---|---|---|
| V1 | ΔV1 | 175 | N | 360 | V360A | 124 | N |
| V1/V2 | ΔV1/2 | 173 | N | 362 | T362A | 102 | N |
| V3 | ΔV3 | 67 | N | 363 | H363A | 97 | N |
| 256 | S256A | 150 | N | 365 | S365A | 1173 | I |
| 262 | N262A[e] | 94 | N | 366 | G366A | 196 | N |
| 265 | L265A | 118 | N | 368 | D368A | 197 | N |
| 268 | E268A | 56 | N | 369 | P369A | 118 | N |
| 276 | N276A[f] | 215 | I | 370 | E370A | 174 | N |
| 288 | L288A | 109 | N | 384 | Y384A | 122 | N |
| 289 | N289A[e] | 58 | N | 386 | N386A[e] | 26 | D |
| 290 | E290A | 122 | N | 388 | T388A | 158 | N |
| 292 | V292A | 137 | N | 389 | Q389A | 77 | N |
| 293 | K293A | 181 | N | 392 | N392A[e] | 5 | D |
| 295 | N295A[e] | 2 | D | 397 | N397A[e] | 148 | N |
| 297 | T297S | 100 | N | 403 | E403A | 122 | N |
| 299 | P299A | 107 | N | 404 | K404A | 32 | D |
| 301 | N301A[f] | 73 | N | 406 | S406A | 33 | D |
| 302 | N302A | 140 | N | 408 | T408A | 40 | D |
| 313 | P313A | 101 | N | 409 | E409A | 62 | N |
| 329 | A329K | 121 | N | 410 | G410A | 86 | N |
| 330 | H330A | 95 | N | 411 | N411A[f] | 136 | N |
| 332 | N332A[e] | 3 | D | 413 | T413S | 120 | N |
| 334 | S334T | 13 | D | 414 | I414A | 146 | N |
| 335 | R335A | 117 | N | 415 | I415A | 72 | N |
| 336 | A336K | 54 | N | 416 | L416A | 2 | D |
| 337 | Q337A | 138 | N | 417 | P417A | 86 | N |
| 339 | N339A[e] | 8 | D | 419 | R419A | 111 | N |
| 340 | N340A | 178 | N | 420 | I420A | 110 | N |
| 343 | K343A | 116 | N | 421 | K421A | 23 | D |
| 344 | Q344A | 227 | I | 445 | C445A | 77 | N |
| 348 | K348A | 146 | N | 450 | T450A | 108 | N |
| 350 | R350A | 99 | N | 458 | G458A | 349 | I |
| 353 | F353A | 22 | D | 463 | S463A | 107 | N |
| 355 | N355A[f] | 155 | N | | | | |

[a] Position refers to gp120 position in HxB2 sequence. The position of an asparagine residue at which the N-linked glycan is implicated in 2G12 binding is underlined.
[b] Mutation XnnnY, where amino acid X at position nnn is substituted by Y; Δ denotes the following amino acid deletions: ΔV1 (amino acids 134 to 154); ΔV1/V2 (amino acids 134 to 154 and 160 to 193); ΔV3 (amino acids 303 to 324).
[c] Apparent affinities were calculated as the antibody concentration at 50% maximal binding. Relative affinities were calculated using the formula [(apparent affinity of wild type)/(apparent affinity of mutant)] × 100%.
[d] Effect on antibody affinity: N, no change; D, decrease; I, increase.
[e] Oligomannose carbohydrate.
[f] Complex carbohydrate.

of D-galactose, D-glucose, or *N*-acetylglucosamine (500 mM). The level of 2G12 binding was determined as described above. Inhibition of 2G12 binding by CVN was measured in an analogous fashion. Following capture of gp120, CVN (0.1 μg/ml) was added to the microtiter plate for 30 min at room temperature prior to the addition of 2G12.

**Generation of recombinant gp120 from CHO cells.** Chinese hamster ovary (CHO) cells, stably transfected with pEE6HCMVgp120GS (obtained from P. Stevens) to secrete recombinant HIV-1 $gp120_{IIIB}$, were cultured in CB2 DMEM Base culture medium (Gibco) supplemented with fetal calf serum (10%), penicillin, and streptomycin. High expression of gp120 was maintained by the addition of methionine sulfoximine (200 μM), whereby cells were grown in the presence or absence of *N*-butyl-deoxynojirimycin (NB-DNJ; 2 mM). gp120 was partially purified using concanavalin A. 2G12 binding was determined following capture of partially purified gp120 onto microtiter plates by using antibody D7324. In this case, the level of 2G12 binding was quantified using fluorescein-conjugated goat anti-human IgG [$F(ab')_2$ specific; diluted 1:1,000; Serotec Ltd.].

**Release of *N*-glycans from SDS-polyacrylamide gel electrophoresis bands.** For high-performance liquid chromatography (HPLC) analyses, the glycans were released directly from sodium dodecyl sulfate (SDS)-polyacrylamide gels containing gp120, following the method of Kuster et al. (25) with some modifications. Briefly, recombinant $gp120_{JR-FL}$ (10 μg) was first reduced by adding 0.25 μl of dithiothreitol (10 M) per sample. Iodoacetamide was then added (final concentration, 10 mM), and samples were incubated in the dark for 30 min at room temperature. Samples were subsequently incubated for 10 min at 70°C and then loaded onto precast 10% bis-Tris gels (Novex). Following electrophoresis, gels were stained with Coomassie blue (for at least 2 h) and destained. Relevant bands were excised, cut into smaller fragments, and frozen on dry ice (2 h). The gel pieces were washed twice with 20 mM $NaHCO_3$ (300 μl; pH 7.0) for 30 min and then with a mixture consisting of equal volumes (300 μl) of acetonitrile and 20 mM $NaHCO_3$ for 60 min to remove any residual SDS. The gel fragments were dried in a vacuum centrifuge and subsequently used for in situ enzymatic digestion to release N-linked glycans for HPLC analysis. For this, peptide-*N*-glycanase F (30 μl; 100 U/ml) was added to the gel fragments and incubated for 12 to 16 h at 37°C. After incubation, samples were centrifuged (2,000 × *g*; 5 min). The supernatants were retained and the glycans were extracted by washing in water (three times; 200 μl) with sonication (30 min), followed by two alternate washes in acetonitrile and water (200 μl). All supernatants were pooled, evaporated to dryness, and redissolved in water (200 μl). Activated AG-50 X12 slurry (200 μl; $H^+$ form) was added to the pooled supernatants and incubated for 5 min at room temperature to remove salts. The mixtures were centrifuged, and the supernatants were filtered through a 0.45-μm-pore-size filter (Millex-LH, hydrophilic polytetrafluoroethylene) and then dried using a vacuum centrifuge.

**Fluorescence labeling of released glycans.** Released glycans were tagged using a 2-aminobenzamide (2AB) labeling kit (Ludger Ltd).

**NP-HPLC.** 2AB-labeled oligosaccharides were dissolved in water (100 μl), and 20-μl aliquots were diluted with 80 μl of acetonitrile for analysis by normal-phase HPLC (NP-HPLC). A calibration ladder of a 2AB-labeled dextran hydrolysate was used to convert the elution times of the gp120 glycans into glucose units (GU) (19). Preliminary structural assignments were then made by comparing the experimental GU values with a database of GU values for standard sugars by

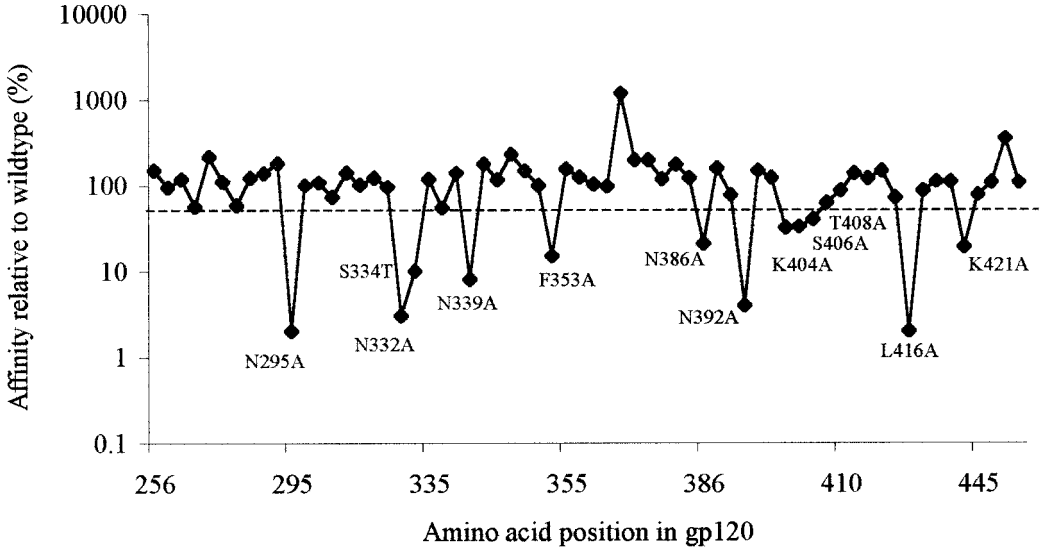


FIG. 1. Apparent affinity of 2G12 for alanine mutants of $gp120_{JR\text{-}CSF}$ relative to that of parent $gp120_{JR\text{-}CSF}$. HxB2 sequence numbering is used (22; Korber et al., HIV Sequence Database, 2001 [http://hiv-web.lanl.gov]). The substitutions that caused a more than 50% (dashed line) reduction in apparent affinity are labeled.

using PeakTime (E. Hart, R. A. Dwek, and P. M. Rudd, unpublished data). Structures were confirmed by exoglycosidase digestions of the 2AB-labeled glycan pool as previously described (19, 53). Aliquots of the total glycan pool were dissolved in sodium acetate buffer (50 mM; pH 5.5) in a total volume of 10 μl. Enzymes were added, and the samples were incubated at 37°C for at least 18 h. The enzymes were removed from the reaction mixture by centrifugation filtration (Micropure-EZ; Millipore Ltd). The samples were subsequently dried in a vacuum centrifuge, dissolved in water (20 μl), and analyzed by HPLC as described above.

## RESULTS

**Alanine scanning mutagenesis of $gp120_{JR\text{-}CSF}$ to identify residues important for 2G12 binding.** Several mutations which disrupt attachment sites for N-linked carbohydrates on gp120 have previously been shown to significantly reduce 2G12 binding to monomeric gp120 (60). However, no systematic mutagenesis analysis has been carried out, and the involvement of extensive regions of gp120 protein surface in the interaction with 2G12 cannot be excluded. Therefore, we decided to carry out extensive alanine scanning mutagenesis. We targeted amino acids predicted to be accessible to antibody from the only available structures of gp120: those of core gp120 complexed to CD4 and the Fab fragment 17b (26, 27).

Mutagenesis was carried out using gp120 from the isolate JR-CSF as the parent. Sixty-three single-amino-acid variants were generated; all of these substituted alanine for the amino acid in the parent gp120 with a small number of exceptions. Where alanine occurs in the parent gp120, it was substituted by lysine. In two cases where threonine and serine form part of an N-linked carbohydrate signal sequence (specifically, T297 and S334), they were substituted by serine and threonine, respectively, to maintain the signal (NXS/T) while altering the residue at these positions. Mutant monomeric gp120s from recombinant pseudovirions were captured onto ELISA wells and probed with various concentrations of 2G12 to generate a binding curve for each mutant. Apparent binding affinities were determined from the concentration of 2G12 at half-maximal binding. The apparent affinity of 2G12 for each mutant gp120 was then related to that for wild-type gp120 (Table 1). Changes in relative affinity greater than 200% were designated as increases, while those below 50% were designated as decreases. Intermediate values were therefore recorded as neutral, i.e., having no or limited effect on 2G12 binding.

The overwhelming majority of alanine substitutions in gp120 had a limited effect on 2G12 binding (Fig. 1). Twelve amino acid substitutions resulted in significant decreases in 2G12 affinity. Five of these substitutions altered triplet sequence motifs (NXS/T) coding for potential N-linked glycosylation sites: N295A, N332A, N339A, N386A, and N392A (Fig. 1). Other substitutions producing lowered affinity for 2G12 were on the silent face of gp120 (S334T, L416A), in the V4 loop (K404A, S406A, T408A), in the coreceptor binding site (K421A), and at the junction between the inner and outer domains of gp120 (F353A).

For those mutants showing decreased affinities for 2G12, we also investigated binding of the human neutralizing antibody b12, which recognizes an epitope overlapping the CD4 binding site (7, 51) (Table 2). N295A and N332A mutants showed essentially unchanged b12 binding affinities. However, N339A, N386A, and N392A mutants all displayed significantly lowered b12 affinities, implying that the substitutions may induce extensive misfolding or conformational perturbation. Therefore, the corresponding carbohydrate chains may not be involved in 2G12 binding. The retention of 2G12 binding by the T388A mutant (Table 1), in which the carbohydrate signal sequence at position 386–388 is eliminated, suggests that the carbohydrate chain at N386 is indeed not involved in 2G12 binding. To further investigate the importance of the carbohydrates at N339 and N392, N→Q substitutions were generated (Table 2). The N339Q mutant bound 2G12 with an affinity similar to that of the parent gp120 and bound b12 with an enhanced affinity.

TABLE 2. Comparison of the effects of select mutations in $gp120_{JR-CSF}$ on the binding of MAbs 2G12 and IgG1 b12

| Mutation[a] | Affinity of antibody for indicated mutant relative to wild type (%) | |
|---|---|---|
| | 2G12 | IgG1 b12 |
| N295A | 2 | 80 |
| N295Q | 4 | 261 |
| N332A | 3 | 75 |
| N339A | 8 | 30 |
| N339Q | 91 | 567 |
| N386A | 21 | 23 |
| N392A | 4 | 4 |
| N392Q | 6 | 142 |
| S334T | 10 | 6 |
| L416A | 2 | 2 |
| F353A | 15 | 29 |
| K421A | 19 | 18 |
| K404A | 32 | 101 |
| S406A | 33 | 60 |
| T408A | 40 | 96 |
| S365A | 1,173 | 667 |
| G458A | 349 | 857 |

[a] Amino acid numbering is based on the HxB2 sequence.

This implies that the carbohydrate chain at position 339 may not be crucial for 2G12 binding but that substitution of Asn with Ala, although not with Gln, disrupts the conformation of the 2G12 (and b12) epitope. In contrast, an N392Q mutant, like the N392A mutant, bound 2G12 with considerably lower affinity than the parent gp120, but bound b12 with unchanged affinity. This is consistent with there being some role for the carbohydrate chain at N392 in 2G12 binding.

Of the remaining mutants showing decreased affinities for 2G12 relative to the parent gp120, four (S334T, L416A, E353A, and K421A) displayed a similar reduction in affinity for b12. The substitutions involved may therefore result in some disruption of global conformation or misfolding. Three substitutions in the V4 loop—K404A, S406A, and T408A—produced very modest decreases in the affinity of 2G12 for gp120 while maintaining b12 affinity.

For two amino acid substitutions, G458A and S365A, significant increases in both 2G12 and b12 binding affinities were observed (Tables 1 and 2). Both of these residues are located in the CD4 binding site of gp120. Mutating these residues thus appears to lead to global conformational changes of gp120 which, hence, may influence the presentation of glycans on the silent face.

The results from the alanine scanning mutagenesis were mapped onto the crystal structure of the complexed gp120 core of $HIV\text{-}1_{HxB2}$ (Fig. 2). This approach was thought to be valid as, although the mutagenesis studies used $gp120_{JR-CSF}$, the structure of the core seems to be highly conserved between isolates (26, 27). One caveat is that the structure of gp120 is that of the core molecule complexed to CD4 and Fab 17b, and some differences between liganded and unliganded core gp120 have been proposed (38). The carbohydrates attached to N295, N332, N339, N386, and N392 lie on either side of the V3 and V4 loops of the outer face of gp120 (Fig. 3). Previous site-specific analysis of N-linked glycosylation of gp120 revealed

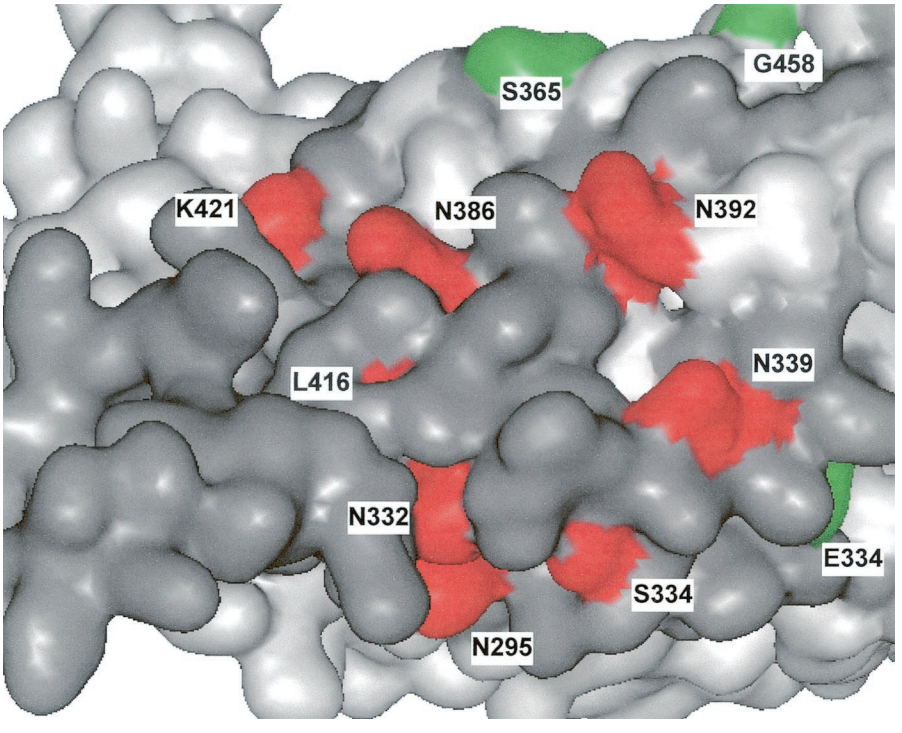


FIG. 2. gp120 structure with amino acids colored to denote the effects of alanine substitutions on 2G12 affinity. The view shows the surface of the C4-V4 face of gp120. Coordinates were taken from the structure of the CD4-liganded core of $gp120_{HxB2}$. Mutations which caused an increase in relative affinity are shown in green, those which did not cause a significant change in affinity are shown in dark grey, and those which caused a decrease in relative affinity are red. For clarity, the V4 loop has been omitted.

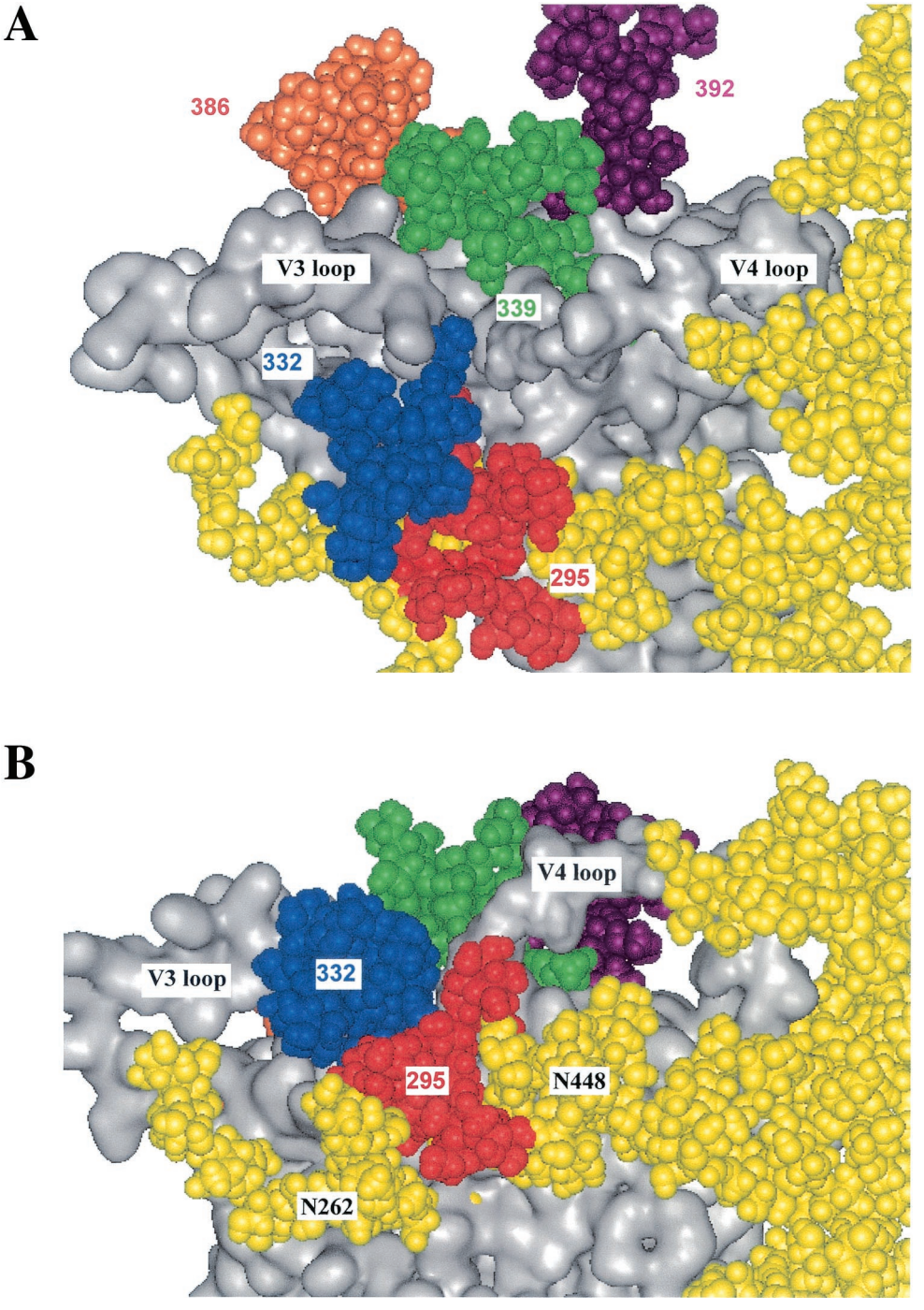


FIG. 3. Location of gp120 N-linked glycans involved in 2G12 binding. The *N*-glycans which are likely to be primarily involved in 2G12 binding are shown in red (*N*-glycan of N295), blue (*N*-glycan of N332), and purple (*N*-glycan of N392). *N*-glycans which influence 2G12 binding but which are not directly involved in binding are shown in green (*N*-glycan of N339) and orange (*N*-glycan of N386). Other carbohydrate chains are shown in yellow. (A) Surface of the C4-V4 face of gp120 viewed from the perspective of the V4 loop. (B) Spatial location of the V3 and V4 loops, which are proposed to extend from the protein surface in the region of the 2G12 epitope. Glycans were modeled onto the core structure of gp120 according to highest population types and lineages, using mass spectrometry (70).

that an assortment of oligomannose glycoforms (70) are attached to these five asparagine residues.

The proximity of the carbohydrate chains to the V3 and V4 loops raises the question as to whether these loops could be involved in 2G12 binding. The very modest effect of V3 deletion on 2G12 binding (Table 1) argues against involvement of the V3 loop. Similarly, deletion of the V1 or V1/V2 regions has modest effects, suggesting that the V1/V2 loop does not sig-

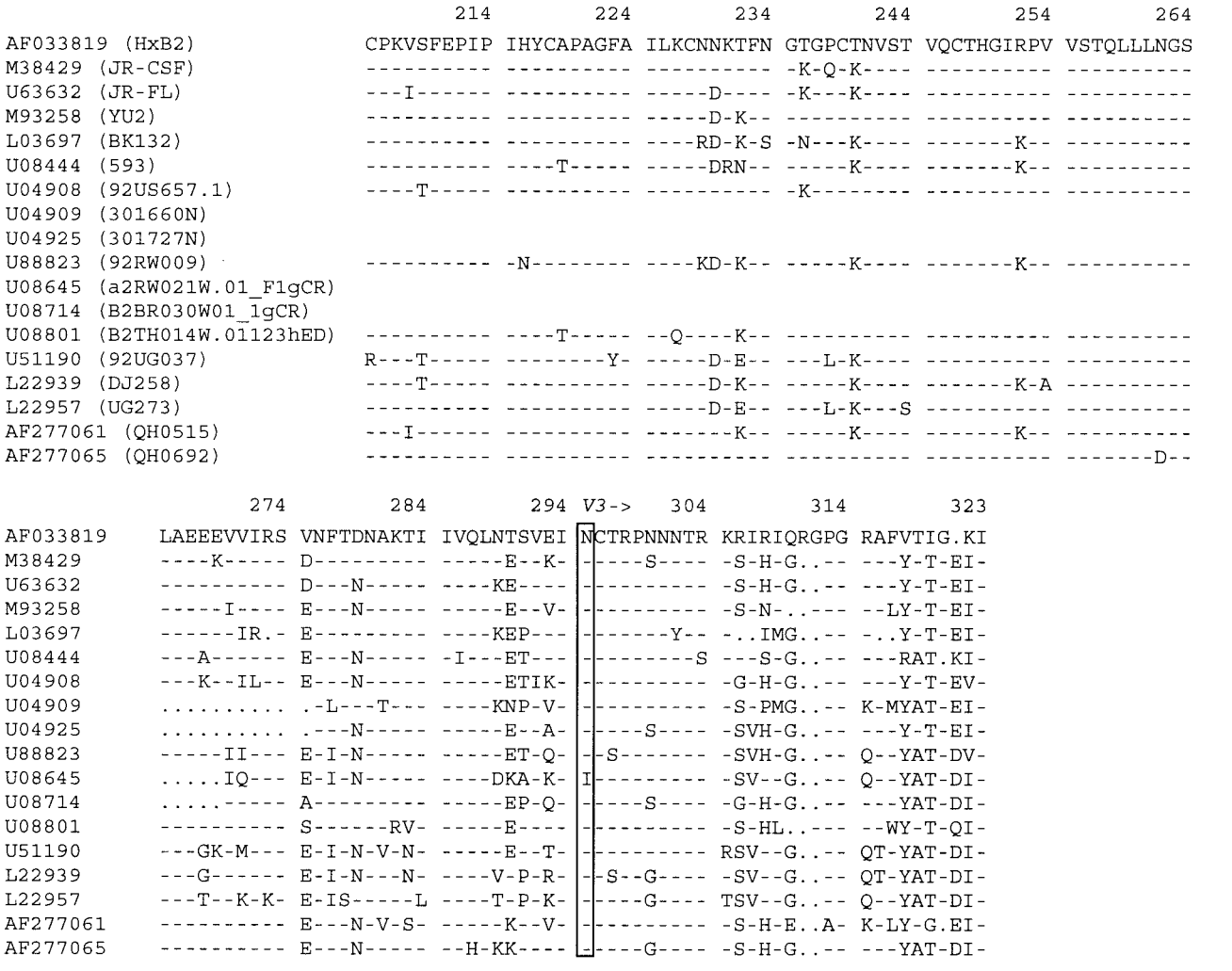


FIG. 4. Sequence alignment of the C2-C5 region of gp120 from HIV-1 isolates neutralized by 2G12. Sequences were obtained from the GenBank database, aligned using ClustalW (http://www.ebi.ac.uk/clustalw/), and formatted for publication using SeqPublish (http://hiv-web.lanl.gov/content/hiv-db/SeqPublish/seqpublish.html). The names of isolates are shown in parentheses. Identical amino acid residues are indicated by dashes; for isolates with the GenBank accession numbers U04909, U04925, U08645, and U8714, only the C2, V3, and C3 regions have been fully sequenced. Rectangular boxes indicate glycosylation sites of *N*-glycans implicated in 2G12 binding.

nificantly impact 2G12 binding. On the other hand, the modest decreases in affinity for 2G12 associated with substitutions in the V4 loop described above suggest that the V4 loop may have some role in 2G12 binding. A V4 loop-deleted mutant was not generated, as previous studies revealed that the envelope of such a mutant is not processed and only gp160 can be immunoprecipitated from transfected cells (68).

**Primary sequence comparison of isolates neutralized by 2G12.** To shed further light on the requirements for the critical carbohydrate chains and to investigate any role for V4, we compared the primary sequences of 18 isolates that have been shown to be effectively neutralized by 2G12. As shown in Fig. 4, the N-linked carbohydrate signal sequences associated with N295, N332, and N392 are highly conserved among the members of the panel, with only one exception at each position. Residue N332, which shows marked sensitivity to alanine substitution in $gp120_{JR\text{-}CSF}$ (Fig. 1), is replaced by a threonine in the isolate U88823, but a new N-linked carbohydrate signal sequence is now associated with N334. In contrast to the above findings, the panel shows slightly more variation at positions 339 and 386 (Fig. 4). Interestingly, the data above also suggest that carbohydrate at these latter positions is dispensable for 2G12 binding.

Comparisons of V4 loop sequences show a great deal of sequence variation in those isolates that are neutralized by 2G12 (Fig. 4). Therefore, it seems unlikely that side chains of V4 loop residues are directly involved in 2G12 binding. However, it remains possible that V4 loop residues, possibly through main chain interactions, are involved in maintaining the relative dispositions of carbohydrate chains for optimal binding of 2G12.

**Glycosidase digestion of gp120 N-linked glycans.** Exoglycosidase and endoglycosidase digestion of the oligomannose glycans of monomeric gp120 gave further insight into the carbohydrate structures that might be required for the 2G12 epitope (Fig. 5). Removal of mannose residues from gp120 by endoH,

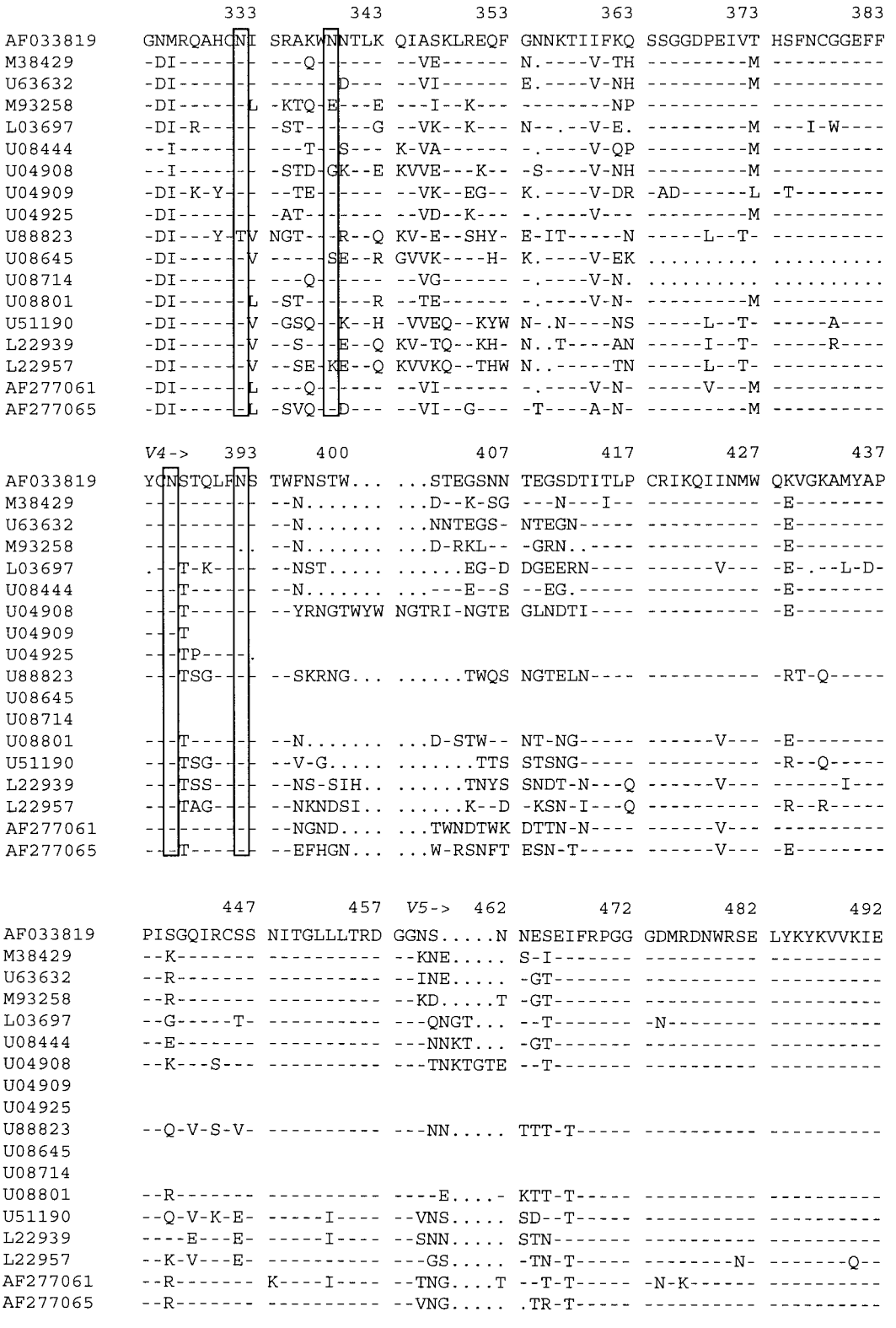


FIG. 4—*Continued.*

leaving only the core asparagine-linked GlcNAc (Fig. 6), dramatically reduced the affinity of 2G12 for gp120 (Fig. 5E). The affinity of the lectin CVN, which binds to Manα1→2Man-linked mannose residues (2–4, 13), was also greatly reduced by endoH treatment (Fig. 5F). In contrast, the affinity of IgG1 b12 was unaffected (Fig. 5D), indicating that endoH treatment does not have global conformational effects on monomeric gp120. Interestingly, there is some residual binding of CVN to

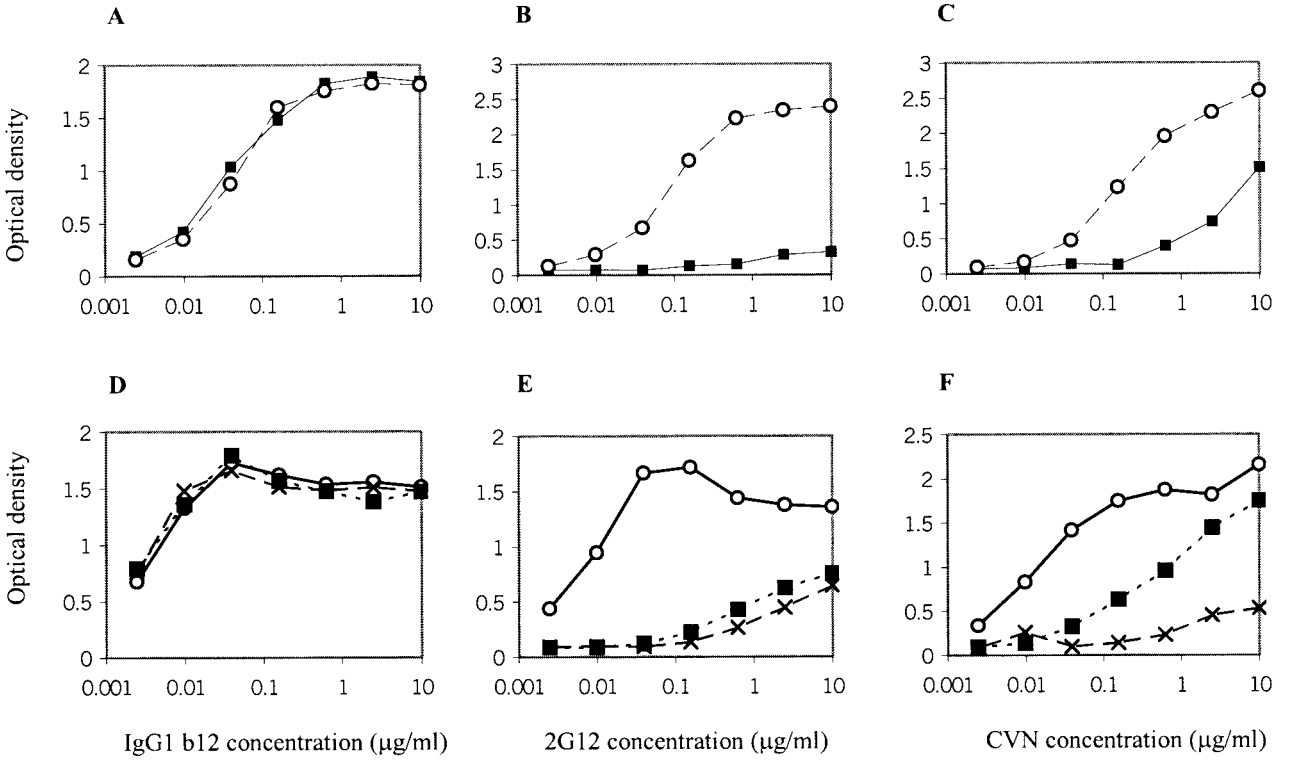


FIG. 5. Binding of IgG1 b12, 2G12, and CVN to enzymatically treated gp120. Top panel: binding of IgG1 b12 (A), 2G12 (B), and CVN (C) to *A. saitoi* mannosidase-treated (■) or untreated (○) gp120. Bottom panel: binding of IgG1 b12 (D), 2G12 (E), and CVN (F) to Jack Bean α-mannosidase-treated (×), endoH-treated (■), or untreated (○) gp120.

endoH-treated gp120, suggesting that some oligomannose structures may be protected from enzyme digestion but they are not able to support 2G12 binding. Removal of either the Manα1→2Man-linked residues by *A. saitoi* mannosidase or Manα1→2,3,6Man-linked residues by Jack Bean mannosidase (Fig. 6) greatly reduced the affinities of both 2G12 (Fig. 5B and E) and CVN (Fig. 5C and F) for gp120, but not that of b12 (Fig. 5A and D).

From these experiments, it appears that the epitope of 2G12 is either formed exclusively of the outer Manα1→2Man residues of oligomannose chains or also involves Manα1→2,3,6Man residues in the context of Manα1→2Man residues.

**Monosaccharide inhibition of the interaction of 2G12 and gp120.** The results from mannosidase digestion strongly suggest that mannose residues are involved in the 2G12 epitope. Consistent with this finding, high concentrations of D-mannose were able to inhibit the interaction of 2G12 and gp120 (Fig. 7A). At similar concentrations, the monosaccharides galactose

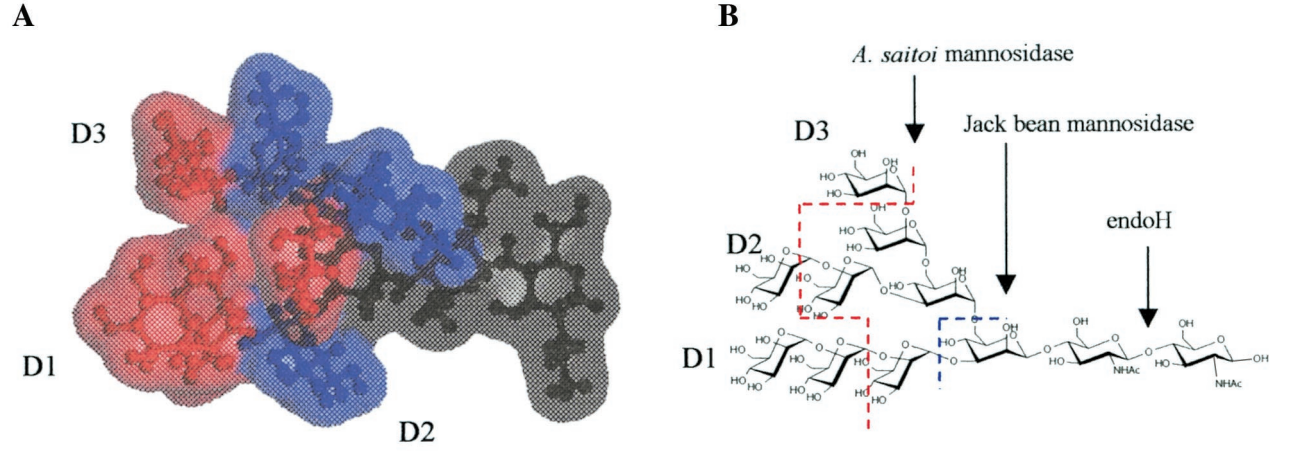


FIG. 6. Structure of $Man_9GlcNAc_2$. (A) Molecular model showing the Manα1→2Man-linked residues (red) and Manα1→2,3,6Man residues (blue) removed by *A. saitoi* mannosidase and Jack Bean mannosidase, respectively. (B) Chemical structure showing cleavage sites for the two mannosidases and endoH. The D1D3 isomer of $Man_8GlcNAc_2$ is derived by removing a single mannose from the D2 arm.

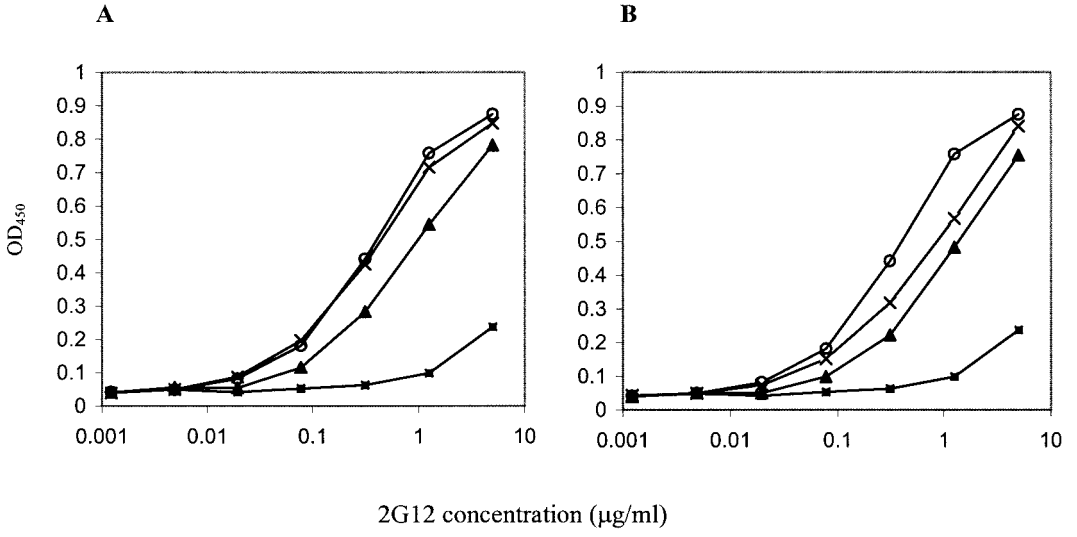


FIG. 7. Monosaccharide inhibition of 2G12 binding to gp120. (A) Binding of 2G12 to $gp120_{JR-FL}$ in the presence of 0 (○), 5 (×), 50 (▲), or 500 (■) mM mannose. (B) Binding of 2G12 to $gp120_{JR-FL}$ in the presence of 500 mM mannose (■), galactose (▲), *N*-acetylglucosamine (×), or buffer (○).

and *N*-acetylglucosamine had little effect on 2G12 binding (Fig. 7B).

**NB-DNJ can inhibit the synthesis of gp120 containing the 2G12 epitope.** The glucose analogue NB-DNJ inhibits the endoplasmic reticulum (ER) glucosidases I and II. These enzymes are responsible for removing the three terminal glucose residues attached to the D1 arm (Fig. 6) of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ that is transferred to the nascent glycoprotein as it enters the ER (20). Inhibition of these enzymes allows gp120 to escape the calnexin-calreticulin folding and quality control system (40) and therefore leads to the secretion of mainly triglucosylated oligomannose-type oligosaccharides (21). The V1 and V2 loops were suggested to have altered conformations under these conditions (15); however, the V3 and V4 loops showed no significant difference in binding to the conformationally sensitive antibodies which were available.

$gp120_{IIIB}$ was produced from CHO cells cultured in the presence of 2 mM NB-DNJ, and binding to 2G12 was assessed by using a fluorescent second antibody. Even at high concentrations (>200 nM) of antibody, no significant interaction between 2G12 and gp120 was found (Fig. 8). The level of gp120 production from cells was not affected by NB-DNJ as determined by Western blotting of cell culture supernatants (data not shown). The results are again indicative of the importance of terminal Manα1→2Man residues in 2G12 binding and, although the effect of the additional glucose residues in inhibiting 2G12 binding may be steric, further suggest that the D1 arm of the oligomannose structure may form a crucial part of the 2G12 epitope.

**CVN can inhibit the binding of 2G12 to gp120.** As described earlier, CVN binds to Manα1→2Man residues and has previously been shown to inhibit the binding of 2G12 to gp120 (13). We also found that incubation of gp120 with excess CVN prevented subsequent binding of 2G12 to gp120 (Fig. 9). However, in a reciprocal experiment, CVN binding to gp120 that was preincubated with excess 2G12 was only slightly reduced compared to binding to gp120 alone (Fig. 9). These data are in agreement with those previously published (13) and are consistent with the notion that 2G12 binds only a subset of the Manα1→2Man termini present on gp120, whereas CVN binds essentially all such residues.

**Analysis of $gp120_{JR-FL}$ N-linked carbohydrates.** The glycosidase experiments described above used gp120 from the JR-FL isolate of HIV-1 expressed in CHO cells. We wished to ascertain that the N-linked carbohydrate composition of this preparation was typical and corresponded to that previously described for $gp120_{SF2}$ (70). Accordingly, a detailed analysis of the glycans was carried out (Fig. 10).

Following enzymatic release and fluorescence labeling of gp120 glycans, over 30 structures were identified by NP-HPLC. Elution times of the glycans were converted to GU by comparison with the elution positions of standard dextran hydrolysate (19). Preliminary assignments were made from a database containing the GU values of standard glycans by using the computer program PeakTime (E. Hart, P. M. Rudd, and R. A. Dwek, unpublished data). The structural assignments were

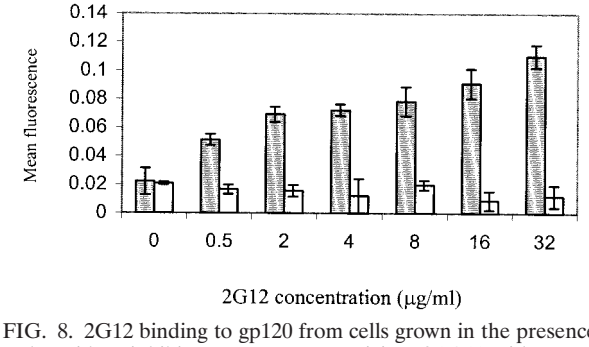


FIG. 8. 2G12 binding to gp120 from cells grown in the presence of the glucosidase inhibitor NB-DNJ. Reactivity of 2G12 with recombinant $gp120_{IIIB}$ expressed in CHO cells, in the presence (open bars) or absence (solid bars) of 2 mM NB-DNJ, is shown.

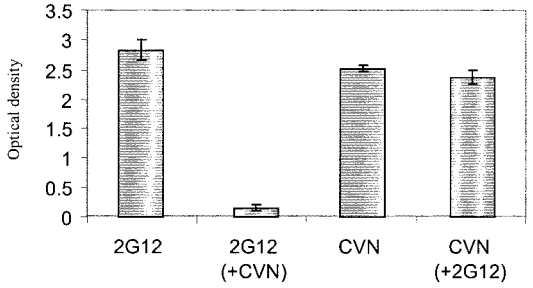


FIG. 9. Inhibition of 2G12 binding to gp120 by CVN. 2G12 and CVN reactivity with $gp120_{JR\text{-}FL}$ (1 μg/ml) alone or gp120 (1 μg/ml) preincubated with CVN (0.1 μg/ml) (+CVN) or with 2G12 (+2G12), respectively.

then confirmed by digestions of the entire glycan pool by using arrays of exoglycosidases (data not shown). The digests were analyzed by NP-HPLC, which provides both monosaccharide sequence and linkage information. $gp120_{JR\text{-}FL}$ contained a mixture of complex and oligomannose glycans (Fig. 10), with the latter structures comprising approximately half of the N-linked glycans. The abundance of oligomannose structures is in agreement with that reported from a previous site-specific analysis of $gp120_{SF2}$ (70).

## DISCUSSION

The human MAb 2G12 is one of the few known broadly neutralizing anti-HIV-1 antibodies. Definition of its epitope at the molecular level may contribute to the design of an immunogen able to elicit 2G12-like antibodies. The antibody has previously been shown to bind an epitope on gp120 that is sensitive to changes in N-linked glycosylation and that does not overlap that of any other known antibody to gp120 (37). Here, site-directed mutagenesis and glycan modification of gp120 have been used to characterize the protein and carbohydrate contributions to the unique 2G12 binding site.

Alanine scanning mutagenesis showed that elimination of the N-linked carbohydrate attachment sequences associated with residues N295, N332, N339, N386, and N392 by N→A substitution produced significant decreases in 2G12 binding affinity to $gp120_{JR\text{-}CSF}$. The N295A and N332A substitutions had specific effects on 2G12 binding to gp120 in that binding of the anti-CD4 binding site antibody b12 was unaffected. In contrast, the N339A, N386A, and N392A substitutions also affected b12 binding, suggesting that they may produce conformational perturbation or protein misfolding, thus bringing into question the involvement of the carbohydrates at these positions in 2G12 binding. Indeed, the retention of 2G12 binding by a T388A mutant in which the carbohydrate attachment sequence at positions 386 to 388 was eliminated confirmed that the carbohydrate chain at N386 is not involved in 2G12 binding. The retention of 2G12 binding by an N339Q mutant similarly argued against the importance of the carbohydrate at N339 in 2G12 binding. Since an N392Q substitution significantly reduced 2G12 binding with little effect on b12 binding, the carbohydrate chain at N392 is likely to be important for 2G12 binding. We also noted that, whereas previous studies had suggested that the carbohydrate at N448 might be important in 2G12 binding, we found this conclusion unlikely for $gp120_{JR\text{-}CSF}$ since a mutant (T450A) in which the carbohydrate signal sequence was eliminated at this position still bound 2G12. Therefore, the mutagenesis studies implicated carbohydrate chains at N295, N332, and N392 as most likely to be important in 2G12 binding.

Previous site-specific analysis of $gp120_{SF2}$ has shown that oligomannose chains are attached at these positions (70). Although we have not repeated the site-specific analysis for $gp120_{JR\text{-}CSF}$, we did show that $gp120_{JR\text{-}FL}$, which is closely related to $gp120_{JR\text{-}CSF}$, has an oligomannose composition similar to that of $gp120_{SF2}$.

Primary sequence comparisons of gp120s known to interact with 2G12 could help to illuminate the relative importance of the residues highlighted by the mutagenesis studies. A comparison of the primary sequences of gp120 from a panel of isolates efficiently neutralized by 2G12 showed that the N-linked carbohydrate signal sequences associated with N295, N332, and N392 are particularly highly conserved. In one of the isolates in which a carbohydrate signal sequence is lost at position 332, it is replaced by another one immediately adjacent. The conservation of the carbohydrate signal sequence associated with N339 is less pronounced than that at the other positions which is, again, consistent with the notion that the carbohydrate at this position is not crucial for 2G12 binding.

One method for the identification of residues crucial to antibody binding to virus is the in vitro or in vivo selection of neutralization escape variants. Previously, we have described the selection of 2G12 neutralization escape variants of $HIV\text{-}1_{JR\text{-}CSF}$ in hu-PBL-SCID mice (severe combined immunodeficiency mice populated with human peripheral blood lymphocytes) (50). The variants all had mutations that eliminated one or both of the carbohydrate signal sequences at positions 339 and 392: N392D, N339S/N392D, N339D, and T341I. These data are in agreement with the mutagenesis studies with respect to N392. For N339, it would appear that the nature of the amino acid substitution at position 339, rather than its ability to eliminate carbohydrate attachment, is important in determining the outcome of its effect on 2G12 binding to gp120.

The carbohydrate chains implicated in 2G12 recognition are close to both the V3 and V4 loops. Involvement of the V3 loop was excluded for $gp120_{JR\text{-}CSF}$, since deletion of the loop had minimal effect on affinity for 2G12. On the other hand, alanine substitutions in the V4 loop produced very modest decreases in 2G12 affinity. The variability of primary sequences in this region, among isolates neutralized by 2G12, argues against a direct role for the V4 loop in 2G12 binding. However, some role for the V4 loop in modulating or maintaining the 2G12 epitope is consistent with all the data. This is further supported by the observation that clade E isolates, most of which have an additional disulfide bond internal to the V4 loop, are not recognized by 2G12 (59; B. T. Korber, B. T. Foley, C. L. Kuiken, S. K. Pillai, and J. G. Sodroski, HIV Sequence Database, 2001 [http://hiv-web.lanl.gov]).

A series of studies carried out on the effects of glycosidases, glycosylation inhibitors, and competing molecules on the interaction of 2G12 and gp120 are all consistent with the critical nature of Manα1→2Man residues in 2G12 binding. (i) Cleavage of specific mannose linkages was found to be sufficient to dramatically reduce 2G12 binding to gp120. EndoH treatment leaving only the core asparagine-linked GlcNAc (Fig. 6), Jack

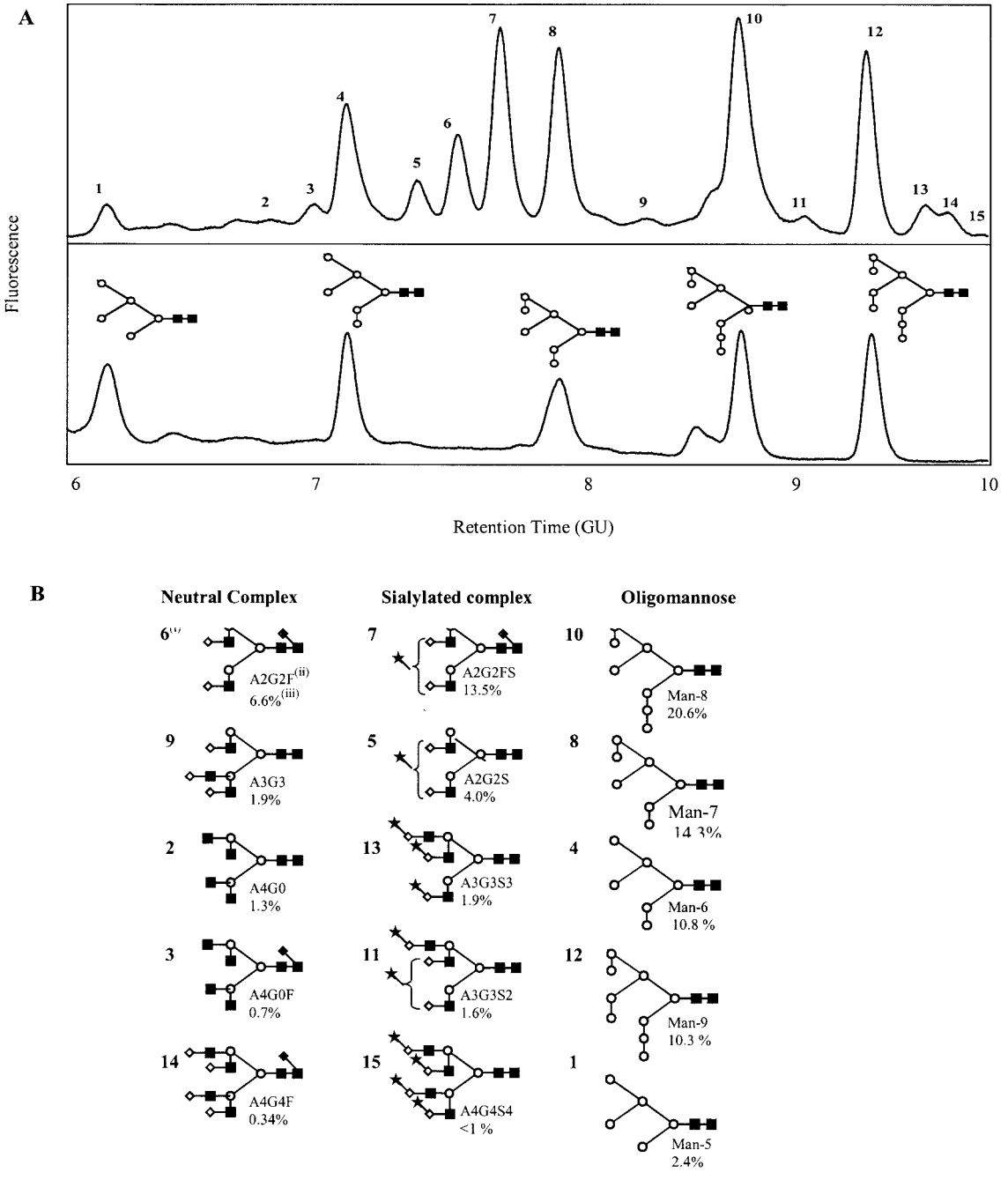


FIG. 10. NP-HPLC of fluorescence-labeled gp120 N-linked glycans. (A) Charged, neutral, and oligomannose structures were released from gp120 by in-gel digestion using PNGase F and resolved by NP-HPLC (top panel). The retention time (GU value) for each peak was compared against a database of known structures. Preliminary assignments, made from the database, were confirmed by exoglycosidase digestions of the entire glycan pool using enzyme arrays. Following comprehensive digestion of complex glycan structures using a panel of exoglycosidases (see Materials and Methods), only oligomannose structures, which represent 58.4% of the total glycans, remained (bottom panel). (B) Symbolic representation of the most abundant sugars is shown together with the peak number (i), nomenclature (ii), and relative abundance (iii) in the glycan pool.

Bean mannosidase treatment leaving $Man_1GlcNAc_2$, and *A. saitoi* mannosidase treatment leaving $Man_5GlcNAc_2$ structures (Fig. 6) were all effective in essentially eliminating 2G12 binding (Fig. 5). *A. saitoi* mannosidase removes a single mannose from each of the D2 and D3 arms and two mannoses from the D1 arm of $Man_9GlcNAc_2$, suggesting that one or more of these residues is critical for 2G12 recognition.

(ii) Mannose inhibited 2G12 binding to gp120 (Fig. 7). In contrast, neither galactose, *N*-acetylglucosamine, nor glucose decreased binding of 2G12. Glucose and mannose are epimers differing only in the stereochemistry of the C2 hydroxyl group. Thus, the precise location of the C2 hydroxyl of mannose may be a determinant of 2G12 specificity.

(iii) gp120 expressed in the presence of NB-DNJ, in which

the D1 arm of oligomannose is blocked by glucose, was unable to bind to 2G12. One caveat here is that NB-DNJ can affect the calnexin-mediated folding of glycoproteins in the ER and has been previously shown to lead to local misfolding of the V2 loop in gp120 (15). In the same study, the V3 and V4 loops were still recognized by a panel of conformationally sensitive antibodies.

(iv) The requirement for specific mannose structures is consistent with the inhibition of 2G12 binding by CVN. CVN binds specifically to the Man$\alpha$1→2Man termini of $Man_8GlcNAc_2$ (D1D3) (Fig. 6) and $Man_9GlcNAc_2$. Preincubation of gp120 with CVN prevents binding of 2G12 to gp120. However, preincubation of gp120 with 2G12 does not significantly reduce the binding of CVN. This suggests that CVN can bind to any suitable oligomannose structure on gp120, whereas 2G12 binds to a selected subset of these oligosaccharides. EndoH treatment, under the conditions described here, partially digests native gp120 glycans. Although some residual CVN binding is observed, there is a complete loss of 2G12 binding. This suggests that the 2G12 epitope contains more than one glycan and removal of any one of these prevents binding.

Another molecule that binds to oligomannose glycans on gp120 is the C-type lectin, dendritic cell specific intracellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) (34, 48). In contrast to other C-type lectins which bind terminal sugars, DC-SIGN binds an internal trisaccharide, the outer trimannose branch point (Man$\alpha$1,3[Man$\alpha$1,6]Man) (14). The data in this paper, and particularly the mannosidase digestions, indicate that 2G12 does not bind the same ligand as DC-SIGN. Nevertheless, DC-SIGN is competitive with 2G12 for binding to gp120 (B. H. Lee, personal communication), presumably via steric interference. Also, it should be noted that 2G12 does not behave as a C-type lectin, since its interaction with gp120 is not perturbed by the presence of EDTA (C. N. Scanlan, unpublished data).

Overall, therefore, our studies support a cluster of mannose residues contributed by up to three different oligomannose chains on the outer face of gp120 as being critical for 2G12 binding, while providing no indication of any direct involvement of protein side chains.

We note that most of the studies described have employed monomeric gp120 produced in CHO or 293T cells, whereas the important functional structure for 2G12-neutralizing activity is the trimeric form of the glycoprotein envelope expressed on viruses that have budded from human lymphocytes (45, 51, 54). It could be argued that the critical glycans on envelope trimers are presented differently from the monomeric preparations. Indeed, differences have been described in the exposure of glycans on monomeric gp120 and native SIV envelope (33). However, the ability of 2G12 to neutralize a broad range of different isolates at nanomolar concentrations corresponding very roughly to the affinities for monomeric gp120 argues that the important structures for 2G12 recognition are similarly presented on monomeric gp120 and HIV envelope trimer. This viewpoint is strengthened by the selection of 2G12 neutralization escape variants with features common to the mutants identified by alanine scanning.

Exclusive recognition of the carbohydrate of a glycoprotein by an antibody is unusual and raises a number of issues. First, such recognition might be thought to be excluded by tolerance mechanisms, as discussed earlier. However, the cluster of tightly packed oligomannose sugars in the region of the 2G12 epitope is unrepresentative of mammalian glycosylation and may, therefore, invoke an antibody response. There are few, if any, mammalian glycoproteins that are extensively mannosylated. Usually when mannose structures are present they are mainly associated with one particular site, e.g., CD2 (69), Thy-1 (42), and tissue plasminogen activator (41). Even when proteins contain oligomannose sugars at more than one site, a survey of N-glycosylated mammalian proteins in the protein database gave no examples of tightly packed sites such as those on gp120 (A. Petrescu and M. R. Wormald, unpublished data). One factor militating against heavy mannosylation of mammalian proteins is effective clearance by mannose receptors (57, 61). Certain pathogens that are heavily mannosylated, such as yeast, are cleared rapidly by this mechanism (39). The second unusual aspect of the 2G12-gp120 interaction is that 2G12 has a high affinity for gp120; typically, the $K_d$ for the interaction is in the nanomolar range. However, the affinities of protein-carbohydrate interactions are generally much weaker (58, 63). The high affinity is also observed for the Fab fragment of 2G12 (R. Pantophlet and D. R. Burton, unpublished data); any avidity effect due to divalent binding of antibody to antigen appears to be weak. One way in which higher affinity may be achieved is if multivalency is exercised within a single antibody combining region, i.e., if the antibody, for instance, bridges between two or more "subsites" corresponding to mannose residues from different carbohydrate chains. The increase in affinity of proteins for carbohydrates achieved through multiple interactions between the sugar and the protein binding site has been discussed (11, 29, 47).

We sought to arrive at a reasonable model for 2G12 interacting with oligomannose chains of gp120 based on our data and considerations of affinity and the unique nature of 2G12. The oligomannose structures located at N295, 332, 339, 386, and 392 are clustered together with the asparagine residues within a 40 by 25 $Å^2$ area on the protein surface (Fig. 3 and 11). N295 and N332 are located in close proximity to one another, as are N339, N386, and N392 (Fig. 11). A model of gp120 based on the structure of core gp120 with modeled oligomannose chains and V3 loop was closely inspected to gain insight into the mobility of the glycans. It appears that the glycan at N332 is likely to be very constrained, and the asparagine residue and the $GlcNAc_2$ (chitobiose) core have extensive interactions with the protein, while the glycan side chain is packed against the V3 loop and the glycan at N295. The mobility of the glycan at N295 is itself expected to be restricted by glycans at N448 and N262. The glycan at N339 is likely to be somewhat restricted in its mobility by interactions of the asparagine residue and the chitobiose core with the protein and of the glycan with the V3 and V4 loops. The presence of glycan at N392 may further constrain the glycan at N339. The glycans at N386 and N392 probably have extensive conformational freedom. The reduced dynamic freedom of the glycans on N295, 332, and 339 is not unprecedented (16, 62), but it is in contrast to that suggested for a number of other glycoproteins studied (65).

In general, sugar recognition by antibodies involves three to four distinct subsites. The shape and topology of these subsites determine the specificity for a particular 3D arrangement of sugars (18). In the case of gp120, it is possible to construct a

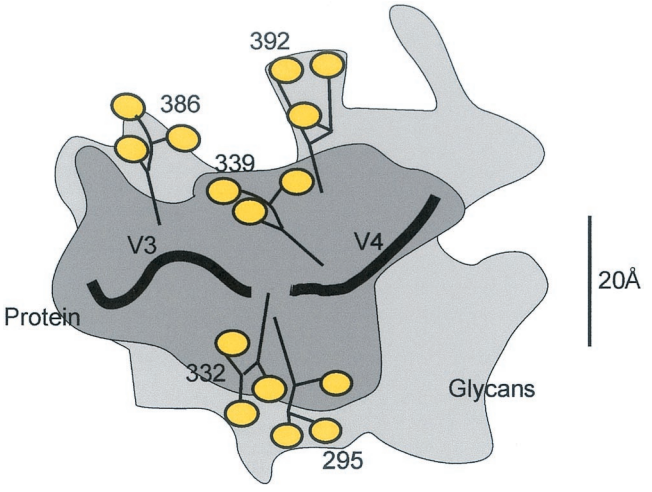


FIG. 11. Schematic model of the approximate locations of the carbohydrate chains on gp120 implicated in 2G12 binding. The dark grey area represents the contour of the protein, and the light grey represents the contour of the glycans. The D1 terminal mannose structures of the glycans attached to asparagines at positions 332, 295, 339, 386, and 392 are shown in yellow. The 20 Å ruler represents the size of a typical antibody binding site.

novel sugar epitope (i.e., nonself) from the tight clustering of the different oligomannose structures on the silent face of gp120. Based on all the experimental data, inspection of the molecular model of gp120, and a typical antibody footprint corresponding to a circle of about 20 Å diameter (Fig. 11), the most likely epitope for 2G12 is provided by the sugars at N295 and N332. When viewed from above, the three terminal mannose residues from the D1, D2, and D3 arms of the N332 carbohydrate and the two terminal mannose residues from the D2 and D3 arms of N295 come together to form a relatively flat surface presenting a contiguous epitope about 15 Å across. The other glycans would then be involved in maintaining the conformation of the glycans at N295 and N332. A less likely alternative epitope would involve mannose residues from carbohydrates at N332 and N339. The terminal two mannose residues on the D3 arm of the sugars at N332 could form an epitope with the three mannose residues on the D3 arm of the sugar at N339. The argument against this alternative is the apparent dispensability of the glycan at N339 in the cases described above. Another possibility that should be considered is that Fab 2G12 recognizes mannose residues from one pair of neighboring glycan chains by the antibody combining site residues and the other pair by residues outside the combining site in a manner similar to that of rheumatoid factor (10).

The challenge from a vaccine development perspective is now to present mannose residues in such a way as to elicit 2G12-like antibodies. This will most likely require first the determination of a crystal structure for 2G12 complexed to its carbohydrate epitope. However, even prior to that, it may be useful to investigate the immunogenicity of oligomannose chains associated on a surface. Furthermore, 2G12 represents an intriguing paradigm for HIV vaccine research. The dense crowding of carbohydrate chains on one face of gp120 has likely given rise to an unusual arrangement of sugars that constitute the epitope of 2G12. There may well be other such unusual carbohydrate epitopes. If strategies can be designed to elicit antibodies to these epitopes, then the silent face, a major defensive strength of the virus against the humoral immune system, could become a major weakness that can be exploited in vaccine design.

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